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Endocannabinoid system activation contributes to glucose metabolism disorders of hepatocytes and promotes hepatitis C virus replication

Li-Jie Sun [a], Jian-Wu Yu [a,*], Lin Wan [a], Xiao-Yu Zhang [a], Yu-Guang Shi [b], Mo-Yang Chen [a]

[a] Department of Infectious Diseases, Second Affiliated Hospital, Harbin Medical University, 246 Xuefu Road, Nangang District, Harbin 150086, China
[b] Scientific Research and Experimental Center, Second Affiliated Hospital, Harbin Medical University, Harbin, China

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SUMMARY

Background: Insulin resistance is highly prevalent in patients with chronic hepatitis C (CHC) and to some extent accounts for fibrosis and reducing viral eradication. Activated cannabinoid 1 receptor (CB1R) signaling has been implicated in the development of phenotypes associated with insulin resistance and steatosis. We investigated the role of the endocannabinoid system in glucose metabolism disorders induced by hepatitis C virus (HCV) replication.

Methods: Human hepatic stellate cells (HSC; LX-2 cells) were co-cultured with Huh-7.5 cells or Huh-7.5 cells harboring HCV replicon (replicon cells). Endocannabinoid levels were then measured by liquid chromatography/mass spectrometry. The expression of CB1R and its downstream glucose metabolism genes in hepatocytes were determined by real-time PCR and Western blot. Glucose uptake by hepatocytes and glucose production were measured. Glucose metabolism tests and measurements of HCV RNA levels and nonstructural protein 5A (NS5A) levels were taken after treatment with CB1R agonist arachidonyl-2-chloroethanolamide (ACEA) or antagonist AM251.

Results: Compared to the co-culture with Huh-7.5 cells, the level of 2-arachidonoylglycerol (2-AG) and the CB1R mRNA and protein levels increased in the co-culture of LX-2 cells with replicon cells. The activation of CB1R decreased AMP-activated protein kinase (AMPK) phosphorylation, inhibited cell surface expression of glucose transporter 2 (GLUT2), and suppressed cellular glucose uptake; furthermore, it increased cyclic AMP response element-binding protein H (CREBH), then up-regulated phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) genes and down-regulated the glucokinase (GK) gene, thus promoting glucose production. Interferon treatment restored the aforementioned changes. CB1R antagonist improved glucose metabolism disorders by an increase in glucose uptake and a decrease in glucose production, and inhibited HCV replication.

Conclusions: HCV replication may not only increase the 2-AG content, but may also up-regulate the expression of CB1R of hepatocytes, then change the expression profile of glucose metabolism-related genes, thereby causing glucose metabolism disorders of hepatocytes and promoting HCV replication. Treatment with CB1R antagonist improved glucose metabolism disorders and inhibited viral genome replication.

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1. Introduction

The hepatitis C virus (HCV) infects approximately 170 million people worldwide, and a proportion of these will develop cirrhosis and hepatocellular carcinoma.[1] Metabolic factors, namely insulin resistance and steatosis, are frequent findings in patients with chronic hepatitis C (CHC). Insulin resistance is frequently observed in non-obese patients, and 36.8% of patients with CHC were shown to have a homeostasis model assessment-insulin resistance (HOMA-IR) index ≥2.5.[2] These metabolic features are independently associated with the severity of liver damage[3] and are negative predictors of a sustained virological response (SVR) after standard antiviral therapy.[4] We have proved that HCV replication may cause a disturbance in glucose metabolism via the silent

* Corresponding author. Tel.: +86 0451 86605614; fax: +86 0451 86605614.
E-mail address: yujianwu45@sina.com.cn (J.-W. Yu).

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information regulator 1 (SIRT1)-AMP-activated protein kinase (AMPK) signaling pathway.[5,6] However the molecular mechanism behind this phenomenon has yet to be explored.

The endocannabinoid system (ECS) consists of cannabinoid receptors, endocannabinoids, and the enzymes involved in their biosynthesis and degradation, and is present both in the brain and peripheral tissues, including the liver.[7] Endocannabinoids include anandamide and 2-arachidonoylglycerol (2-AG). There are two G protein-coupled cannabinoid receptors, CB1 and CB2.[8] Endocannabinoids acting through cannabinoid 1 receptor (CB1R) have a strong anabolic effect and the activation of hepatic CB1R increases the activity of lipogenic and gluconeogenic transcription factors.[9] Thus, CB1 activation is associated with insulin resistance and dyslipidemia. Additionally, up-regulation of the hepatic CB1R may contribute to chronic liver inflammation, fibrosis, and cirrhosis induced by obesity, alcoholism, and viral hepatitis.[10]

Several lines of investigation have suggested the crucial role of the hepatic cannabinoid system, which appears to be mediated via activation of cannabinoid receptors, in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH).[11,12] Two recent studies suggested that the expression of CB1R is up-regulated in liver samples from CHC patients,[13] and that the activation of the cannabinoid system is crucial for lipid accumulation in HCV replicon cells.[14] Antagonism of peripheral hepatic CB1R improves insulin sensitivity and liver carbohydrate and lipid metabolism in cultured mouse liver slices.[15] However, it is unclear whether insulin resistance promotes the development of CHC via activation of the endocannabinoid/cannabinoid receptor signaling pathway. We speculated that the cannabinoid signal pathway may play an important role in hepatic glucose disorders in HCV infection. In this study we investigated the role of the endocannabinoid system in insulin resistance induced by HCV infection using a co-culture system of Huh-7.5 cells harboring HCV replicon and hepatic stellate cells (HSC).

2. Materials and methods

2.1. Cell culture

Huh-7.5 cells and Huh-7.5 cells harboring HCV replicon (hereafter referred to as replicon cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone), 100 units/ml penicillin (Hyclone), and 100 μg/ml streptomycin (Hyclone) at 37 °C in a humidified 5% CO_2 incubator and passaged every 3 days by trypsinization. Replicon cells harboring a full-length HCV-1b RNA replicon derived from pON/C-5B, autonomously replicate to express all the HCV proteins (the core protein, E1, E2, p7, NS2, NS3 to NS5B).[6] Replicon cells were treated with 1000 IU/ml of alpha interferon (IFN; Sigma Chemical, St. Louis, MO, USA) for 10 days to eliminate HCV replication.

HSC (LX-2 cells) were purchased from the cell bank of the Chinese Academy of Science. The Huh-7.5 cells and replicon cells and replicon cells with IFN treatment were then cultured in serum-free medium overnight (serum starvation) and co-incubated with LX-2 cells at a density of 5×10^7 cells/l. The ratio of hepatocytes to LX-2 cells was 4:1. LX-2 cells were separated from hepatocytes using a 0.2-μm Anopore semipermeable membrane (Nalge Nunc., Naperville, IL, USA). Following 24 h culture in DMEM supplemented with 10% FBS, the cells were washed with phosphate buffered saline (PBS) and cultured for another 24 h in serum-free DMEM. The medium was then collected to measure endocannabinoid levels.

2.2. Measurement of anandamide and 2-AG

Anandamide and 2-AG levels were determined by liquid chromatography (LC)/mass spectrometry (MC) as described previously.[16] Lipid extracts of the medium were made by first adding 9.0 ml of a 1:1 mixture of methanol and acetonitrile to 1.0 ml medium. The samples were spiked with 1 nmol of *d*-8-anandamide (Deva Biotech, Hatboro, PA, USA) and 10 nmol *d*-8-2-AG (Deva Biotech) as internal standards. This solution was vortexed for 1 min then underwent centrifugation at 19 000 × *g* at 24 °C for 20 min. The supernatant was fractionated by reverse-phase high performance LC on an ODS column (Supelcosil, 5 μm, 4.6 mm × 15 cm), using a mobile phase of methanol/water/acetic acid (85:15:0.03 vol/vol/vol) at a flow rate of 1 ml/min. This was followed by in-line MS analysis on a Micromass Quattro II mass spectrometer equipped with an atmospheric pressure, chemical ionization source. Two selected ions were monitored simultaneously: selected on monitoring 387.2 for *d*-8-2-AG ions as the internal standard, selected on monitoring 379.2 for 2-AG ions, selected on monitoring 356.2 for *d*-8-anandamide ions as the internal standard, and selected on monitoring 348.2 for anandamide ions.

2.3. Glucose uptake by hepatocytes

The Huh-7.5 cells and replicon cells and replicon cells with IFN treatment were cultured in 12-well plates for the glucose uptake assay. After serum starvation overnight in 0.1% bovine serum albumin–DMEM, the cells were subsequently incubated in 1 ml of PBS containing 100 nM insulin for 30 min at 37 °C. After washing with PBS, cells were incubated in 1 ml of PBS containing 1 μCi of 2-deoxy-D-[^{3}H] glucose/ml (ICN, Costa Mesa, CA, USA) for 5 min. The reaction was stopped by adding 0.1 mM cytochalasin B on ice. The cells were washed with ice-cold PBS and solubilized in 0.4 ml of NaOH (0.05 M). The incorporated radioactivity was measured in 4 ml of scintillation fluid by liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA, USA).

2.4. Glucose production assay

The Huh-7.5 cells and replicon cells and replicon cells with IFN treatment were cultured in 12-well plates for the glucose production assay. Culture medium was replaced with glucose production buffer consisting of glucose-free DMEM, without phenol red, supplemented with a gluconeogenic substrate (2 mM sodium pyruvate and 20 mM sodium lactate). After 24 h of incubation, the medium was collected, and the total glucose concentration was measured using the Glucose Assay Kit II (Biovision, CA, USA) and normalized to the cellular protein content. As the baseline of glucose production, glucose-free DMEM with neither sodium pyruvate nor sodium lactate was used. Glucose production via gluconeogenesis equals the total glucose production minus the baseline glucose production.

2.5. Real-time PCR analysis of mRNA levels of CB1 and glucose metabolism-related genes

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. cDNA was generated using the Quanti Tect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR Premix Ex Taq (Takara Bio, Kyoto, Japan) using SYBR green chemistry in an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for normalization. Table 1 lists the PCR primers for the

Table 1
Sequences of primers used for the real-time PCR

Genes	Forward 5′–3′	Reverse 5′–3′	PCR products (bp)
CB1R	CAGAAGAGCATCATCCACACGTCTG	ATGCTGTTATCCAGAGGCTGCGCAGTGC	338
DAGLα	CCTCTTCAACCTGGACAGCAA	GGGCCCTCAGCGTAGTCA	245
DAGLβ	TTCCAAGGAGTTCGTGACTGC	TTGAAGGCCTTGTTGTCGCC	207
MGL	ATGCAGAAAGACTACCCTGGGC	TTATTCCGAGAGAGCACGC	135
GLUT1	TGAACCTGCTGGCCTTC	GCAGCTTCTTTAGCACA	399
GLUT2	TGG GCTGAGGAAGAGACTGT	AGAGACTGAAGGATGGCTCG	461
GK	GCCTCCCAAAGCATCTACCTC	GCTCCACTGCCCCTCCTCACC	444
G6Pase	CCTGGGGCTGGCTCTCAACTC	AATAGTAGTCCTCCTCAATCC	309
PEPCK	CCAGGCAGTGAGGGAGTTTCT	ACTGTGTCTCTTTGCTCTTGG	217
GAPDH	GACAACTTTGGCATCGT	ATGCAGGGATGATGTTCTGG	133

CB1R, cannabinoid 1 receptor; DAGL, diacylglycerol lipase; MGL, monoglyceride lipase; GLUT, glucose transporter; GK, glucokinase; G6Pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

amplification of monoglyceride lipase (MGL) and diacylglycerol lipase alpha (DAGLα) and beta (DAGLβ) of LX-2 cells; CB1R, glucose transporter 1 (GLUT) and 2 (GLUT2), glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) of hepatocytes; and GAPDH. PCR conditions were as follow: 95 °C for 30 s, 58 °C for 30 s, and extension at 72 °C for 45 s; 40 cycles. The relative gene expression analysis was carried out using SDS 3.1 software (Applied Biosystems).

2.6. Western blot

All the cells in the co-culture system were lysed in buffer (20 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Cell debris was removed by centrifugation at 14 000 × *g* for 15 min at 4 °C, and the cell supernatant was used for Western blotting. Protein quantification was carried out using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Fifty micrograms of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with specific antibodies (anti-phospho-AMPKα (Thr172) monoclonal antibody, anti-AMPKα antibody (Cell Signaling Technologies, Beverly, MA, USA), anti-HCV NS5A monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclic AMP response element-binding protein H (CREBH) antibody (Thermo Fisher Scientific, USA), and anti-CB1 receptor (Affinity BioReagents, Golden, CO, USA)), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology). The respective protein bands were visualized by the enhanced chemiluminescence detection system (Perkin Elmer, Waltham, MA, USA). Protein loading was normalized by probing with β-actin antibody (Santa Cruz Biotechnology).

2.7. Cell viability

The effect of CB1R agonist arachidonyl-2-chloroethanolamide (ACEA; Tocris Bioscience, Avonmouth, Bristol, UK) and antagonist AM251 (Tocris Bioscience) on cell viability was investigated. The cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24 h culture, the cells were treated with ACEA (1–10 nM) or AM251 (1–10 nM) in the absence of G418. After incubation for 72 h, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

2.8. HCV RNA level

The HCV RNA level was quantified by real-time PCR (in an ABI Prism 7000 Real-Time Thermocycler), with the use of HCV analyte-specific reagents (ASRs, Abbott) from the Pathology Clinical Laboratory at Saint Louis University.

2.9. Statistical analysis

Results were expressed as the mean ± standard deviation. Significance was analyzed using the Student's *t*-test, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effect of HCV replication on endocannabinoid levels

Compared to those co-cultured with Huh-7.5 cells, cells in the co-culture of LX-2 cells with replicon cells showed a significant difference. The level of 2-AG (80.5 ± 20.1 vs. 30.0 ± 5.0 pmol/10^6 cells, $t = 5.941$, $p < 0.01$) increased, which was restored by IFN treatment (32.9 ± 6.2 vs. 30.0 ± 5.0 pmol/10^6 cells, $t = 0.909$, $p > 0.05$) (Figure 1). The mRNA level of DAGLβ (the enzyme responsible for the synthesis of 2-AG) increased (2.4 ± 0.5 vs. 1.0 ± 0.3, $t = 5.881$, $p < 0.01$), and was restored by IFN treatment (0.9 ± 0.2 vs. 1.0 ± 0.3, $t = 0.679$, $p > 0.05$). The mRNA levels of DAGLα and MGL (the enzymes responsible for the selective degradation of 2-AG) decreased significantly (DAGLα 0.4 ± 0.1 vs. 1.0 ± 0.3, $t = 4.648$, $p < 0.01$; MGL 0.5 ± 0.2 vs. 1.0 ± 0.3, $t = 3.397$, $p < 0.01$), and they were restored by IFN treatment (DAGLα 0.9 ± 0.2 vs. 1.0 ± 0.3, $t = 0.679$, $p > 0.05$; MGL 0.9 ± 0.2 vs. 1.0 ± 0.3, $t = 0.679$, $p > 0.05$) (Figure 1). No significant difference was seen in anandamide level between co-cultured with Huh-7.5 cells and replicon cells (0.13 ± 0.03 vs. 0.15 ± 0.05 pmol/10^6 cells, $t = 0.840$, $p > 0.05$).

Anandamide and 2-AG were not detected in the single culture medium from LX-2 cells or Huh-7.5 cells or replicon cells.

3.2. Effect of HCV replication on CB1R levels

Compared to those co-cultured with Huh-7.5 cells, the expression level of CB1R protein of hepatocytes (0.8 ± 0.2 vs 0.3 ± 0.1, t = 5.477, p < 0.01) increased in the cells co-cultured with LX-2 cells with replicon cells, and it was restored by IFN treatment (0.4 ± 0.1 vs 0.3 ± 0.1, t=1.577, p > 0.05) (Figure 2). The mRNA level of CB1R (3.3 ± 0.7 vs 1.0 ± 0.2, t = 7.739, p < 0.01) increased, and was restored by IFN treatment (0.9 ± 0.2 vs 1.0 ± 0.2, t=0.866, p > 0.05) (Figure 3).

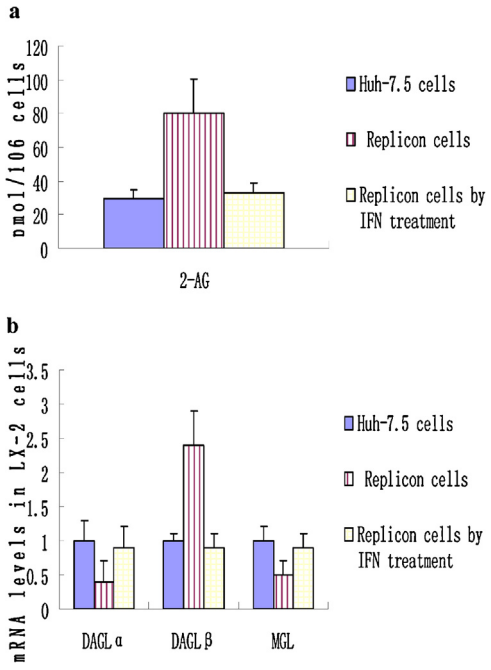

Figure 1. Effect of HCV replication on the endocannabinoid system. (a) Level of 2-AG. (b) Transcriptional levels of the enzymes responsible for the biosynthesis and degradation of 2-AG. The 2-AG level was measured by liquid chromatography/mass spectrometry. The transcriptional levels of the enzymes responsible for the biosynthesis and degradation of 2-AG were examined by real-time quantitative PCR. Each data point represents the mean ± standard deviation of six individual experiments. (Abbreviations: 2-AG, 2-arachidonoylglycerol; IFN, interferon; DAGL, diacylglycerol lipase; MGL, monoglyceride lipase.)

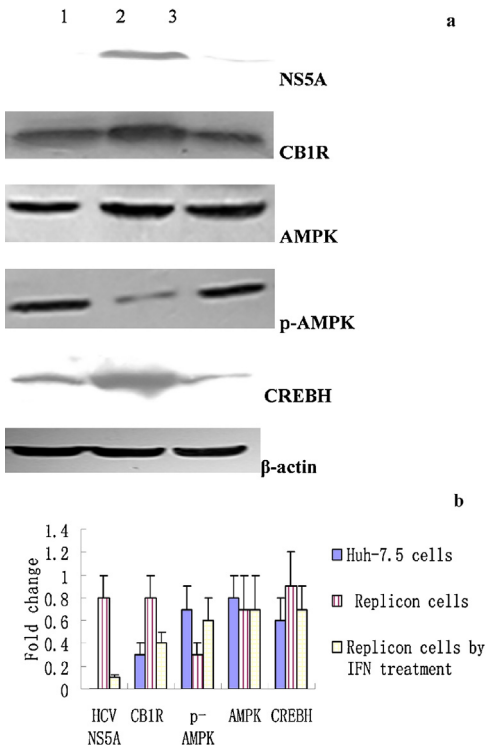

Figure 2. Effect of HCV replication on the expression of CB1R, AMPK, phospho-AMPK, and CREBH protein. (a) The expression of NS5A, CB1R, AMPK, phospho-AMPK, and CREBH protein by Western blot: lane 1, Huh-7.5 cells; lane 2, replicon cells; lane 3, replicon cells with IFN treatment. (b) Signal quantification by densitometry of NS5A, CB1R, AMPK, phospho-AMPK, and CREBH protein normalized to β-actin expression; the data shown are the mean ± standard deviation of six independent experiments. (Abbreviations: CB1R, cannabinoid 1 receptor; AMPK, AMP-activated protein kinase; CREBH, cyclic AMP response element-binding protein H; NS5A, nonstructural protein 5A; IFN, interferon.)

3.3. Effect of HCV replication on the expression of AMPK, phospho-AMPK, and CREBH protein

HCV nonstructural protein 5A (NS5A) was detected in replicon cells by Western blot, and not detected in Huh-7.5 cells or replicon cells with IFN treatment (Figure 2). Compared to Huh-7.5 cells, in replicon cells, the expression level of phospho-AMPK protein decreased (0.3 ± 0.1 vs. 0.7 ± 0.2, $t = 4.382$, $p < 0.01$), and its level was restored by IFN treatment (0.6 ± 0.2 vs. 0.7 ± 0.2, $t = 0.866$, $p > 0.05$); the expression level of CREBH protein increased (0.9 ± 0.3 vs. 0.6 ± 0.2, $t = 2.038$, $p < 0.05$), and its level was restored by IFN treatment (0.7 ± 0.2 vs. 0.6 ± 0.2, $t = 0.866$, $p > 0.05$). There was no difference in expression level of AMPK protein between replicon cells and Huh-7.5 cells (0.8 ± 0.2 vs. 0.7 ± 0.3, $t = 0.679$, $p > 0.05$) (Figure 2).

3.4. Effect of HCV replication on glucose uptake and glucose production

Compared to Huh-7.5 cells, in replicon cells, the glucose uptake level of hepatocytes decreased (cpm 4500 ± 500 vs. $20\,000 \pm 4600$, $t = 8.253$, $p < 0.01$), and it was restored by IFN treatment (cpm $19\,000 \pm 3500$ vs. $20\,000 \pm 4600$, $t = 0.424$, $p > 0.05$) (Figure 3); the glucose production level of hepatocytes increased (2.6 ± 0.5 vs. 1.0 ± 0.2, $t = 7.278$, $p < 0.01$), and IFN treatment canceled the enhanced glucose production (1.1 ± 0.3 vs. 1.0 ± 0.2, $t = 0.679$, $p > 0.05$) (Figure 3).

3.5. Effect of HCV replication on mRNA levels of glucose metabolism-related genes

Compared to Huh-7.5 cells, in replicon cells, the mRNA level of GLUT2 decreased (0.3 ± 0.1 vs. 1.0 ± 0.2, $t = 7.668$, $p < 0.01$), and it was restored by IFN treatment (0.9 ± 0.3 vs. 1.0 ± 0.2, $t = 0.679$, $p > 0.05$). The mRNA level of GK decreased (0.5 ± 0.1 vs. 1.0 ± 0.2, $t = 5.477$, $p < 0.01$) and the mRNA level of PEPCK (2.6 ± 0.6 vs. 1.0 ± 0.3, $t = 5.842$, $p < 0.01$) and G6Pase (2.5 ± 0.5 vs. 1.0 ± 0.2, $t = 6.822$, $p < 0.01$) increased, and the levels were restored by IFN treatment (GK, 0.9 ± 0.3 vs. 1.0 ± 0.2, $t = 0.577$, $p > 0.05$; PEPCK, 1.1 ± 0.3 vs. 1.0 ± 0.3, $t = 0.679$, $p > 0.05$; G6Pase, 1.1 ± 0.3 vs. 1.0 ± 0.2, $t = 0.679$, $p < 0.01$). There was no significant difference in GLUT1 mRNA level between replicon cells and Huh-7.5 cells (0.9 ± 0.2 vs. 1.0 ± 0.3, $t = 0.679$, $p > 0.05$) (Figure 3).

3.6. Cell viability of replicon cells after addition of ACEA and AM251

Since it has been reported that the proliferation of the HCV genomic replicon is dependent on host cell growth, we examined the effect of CB1R agonist ACEA or antagonist AM251 on the cell number and viability of replicon cells by trypan blue dye exclusion test. The cytotoxic effect of ACEA or AM251 was not observed in the cell viability test (Figure 4).

3.7. Effect of CB1 receptor signaling on glucose uptake and glucose production

In order to study the effect of CB1 receptor signaling on glucose uptake and glucose production, we treated replicon cells or Huh-7.5 cells with CB1R agonist ACEA (10 nM) or antagonist AM251 (10 nM) and measured glucose uptake levels and glucose production levels. After 48 h of treatment with AM251, glucose uptake levels increased rapidly and equaled levels of parental Huh-7.5

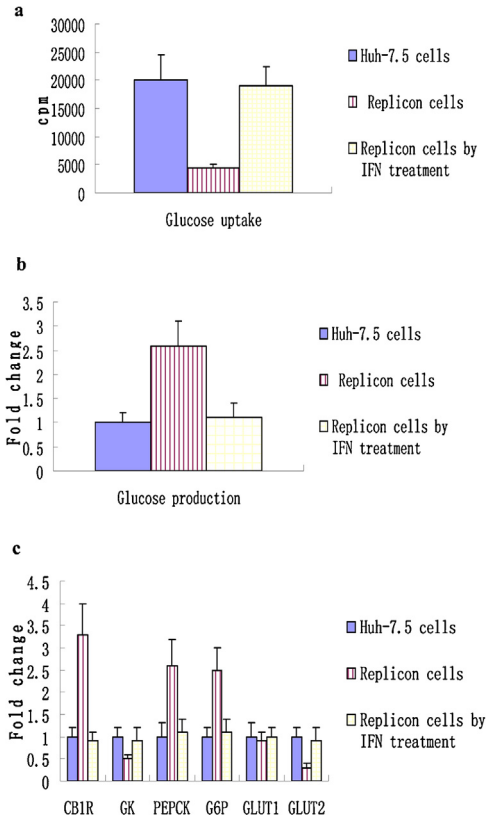

Figure 3. Effect of HCV replication on glucose metabolism. (a) Effect of HCV replication on glucose uptake. (b) Effect of HCV replication on glucose production. (c) Effect of HCV replication on mRNA levels of CB1R and its downstream glucose metabolism-related genes. The level of glucose uptake was measured by a liquid scintillation counter. The level of glucose production was measured using a commercial kit. The transcriptional level of CB1R and its downstream glucose metabolism-associated genes was examined by real-time quantitative PCR. The data shown are the mean ± standard deviation of six independent experiments. (Abbreviations: IFN, interferon; cpm, count per min; CB1R, cannabinoid 1 receptor; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase; GLUT, glucose transporter.)

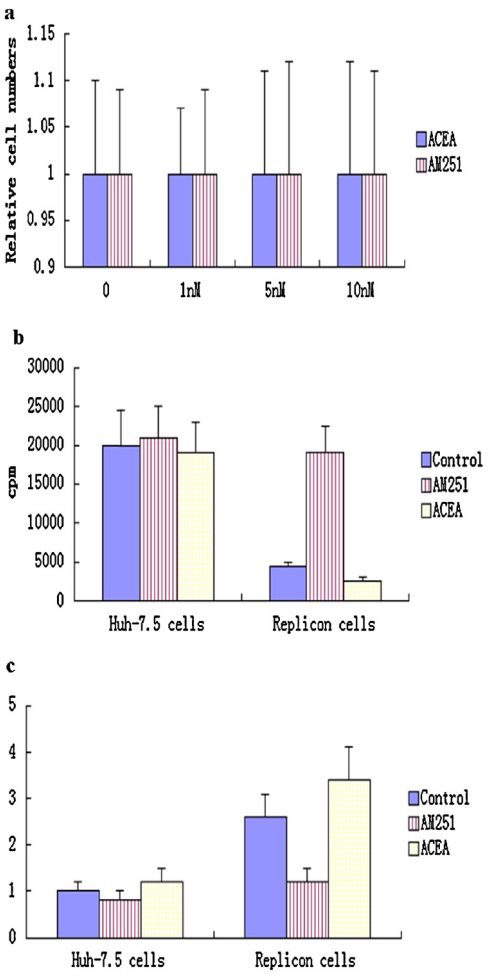

Figure 4. Effect of CB1 receptor signaling on glucose metabolism. (a) Cell viability of replicon cells after the addition of ACEA and AM251. (b) Effect of CB1 receptor signaling on glucose uptake. (c) Effect of CB1 receptor signaling on glucose production. The indicated concentrations of ACEA and AM251 were added to the culture of replicon cells for 72 h. Cell viability was evaluated by trypan blue dye exclusion assay. Replicon cells and Huh-7.5 cells were treated with CB1R agonist ACEA (10 nM) or antagonist AM251 (10 nM) for 48 h. The glucose uptake level and glucose production level were measured. The data shown are the mean ± standard deviation of six independent experiments. (Abbreviations: ACEA, arachidonyl-2-chloroethanolamide; CB1R, cannabinoid 1 receptor.)

cells (Figure 4) and glucose production levels reduced dramatically and equaled levels of parental Huh-7.5 cells (Figure 4), whereas the agonist had the opposite effect.

3.8. Effect of CB1 receptor signaling on HCV replication

To investigate the effect of CB1 receptor signaling on HCV genome replication, we analyzed levels of HCV RNA and HCV NS5A at various time points following treatment of replicon cells with ACEA and AM251. The levels of HCV RNA and NS5A reduced after 72 h treatment with AM251, whereas they increased after 72 h treatment with ACEA (Figure 5).

4. Discussion

The present study provides evidence of the involvement of endocannabinoids acting at hepatic CB1R in the development of HCV-induced liver glucose metabolism disorders. Several findings support this notion. First, HCV replication induced the activation of the hepatic endocannabinoid system, as reflected in the up-regulation of both CB1 receptor expression and 2-AG levels in the co-culture system. Second, treatment with CB1R blockade improved glucose metabolic disorders and inhibited viral genome replication. Third, the aforementioned metabolic disorders were selectively suppressed when HCV replication was eliminated by IFN treatment.

It is well established that the endocannabinoid system contributes to the control of lipid and glucose metabolism. CB1 receptors have been shown to mediate both alcohol- and diet-induced hepatic steatosis and insulin resistance by up-regulating the lipogenic transcription factor sterol regulatory element-binding protein 1c and increasing de novo fatty acid synthesis down-regulating transcription factor carnitine palmitoyl transferase 1 and decreasing fatty acid oxidation.[17] Endocannabinoids contribute to diet-induced insulin resistance in mice via hepatic CB1-mediated inhibition of insulin signaling and clearance, and blockade of CB1 receptors enhances insulin sensitivity.[18] Furthermore, in well-characterized cohorts with CHC, daily cannabis use is significantly associated with the progression of fibrosis and the development of severe steatosis and fibrosis.[19] However, little information is available regarding the downstream signaling pathway involved in this process. In view of the association between endocannabinoid physiology and glucose metabolism, we were interested to explore the role that HCV replication plays in the CB1R signaling pathway and the underlying basis.

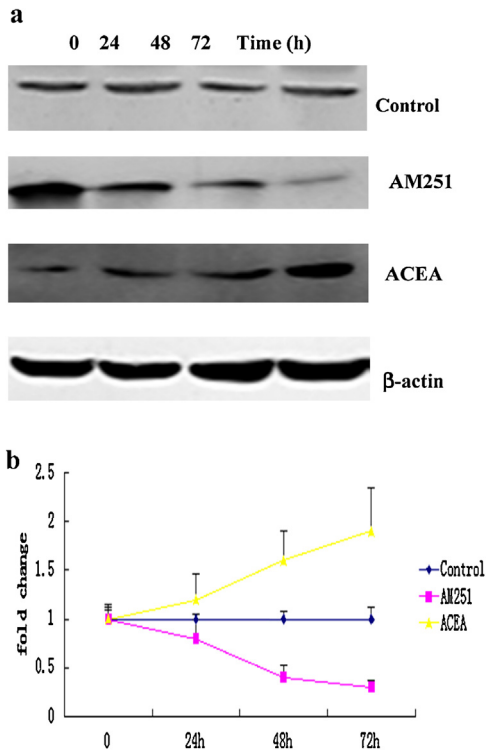

Figure 5. Effect of CB1 receptor signaling on HCV replication. (a) Effect of CB1 receptor signaling on HCV NS5A protein. (b) Effect of CB1 receptor signaling on HCV RNA levels. ACEA or AM251 was added to the medium of replicon cells at a concentration of 10 nM and incubated for 72 h. Replicon replication was assessed by immunoblotting for NS5A and HCV RNA levels at various time points. (Abbreviations: HCV, hepatitis C virus; NS5A, nonstructural protein 5A; RNA, ribonucleic acid; ACEA, arachidonyl-2-chloroethanolamide.)

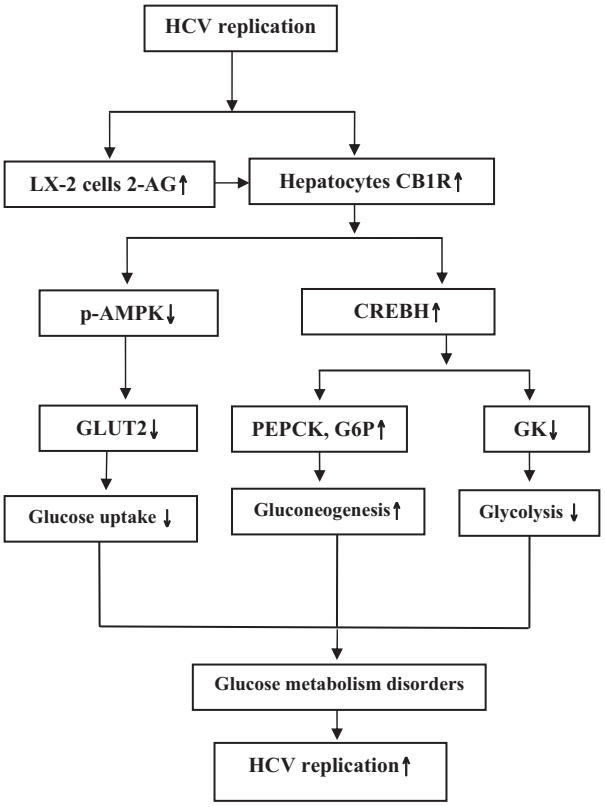

Figure 6. Schematic presentation of the CB1R signaling pathway induced by HCV replication for glucose metabolism disorders of hepatocytes. HCV replication may not only increase the 2-AG content secreted by LX-2 cells, but may also up-regulate the expression of CB1R of hepatocytes, then change the expression profile of glucose metabolism-related genes, thereby causing metabolism disorders of hepatocytes and promoting HCV replication. An arrowhead pointing upwards (↑) represents activation, an arrowhead pointing downwards (↓) represents the repression of activity. (Abbreviations: CB1R, cannabinoid 1 receptor; HCV, hepatitis C virus; 2-AG, 2-arachidonoylglycerol; p-AMPK, phospho-AMP-activated protein kinase; CREBH, cyclic AMP response element-binding protein H; GLUT, glucose transporter; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase; GK, glucokinase.)

The biosynthesis and degradation of anandamide and 2-AG occur through distinct pathways. Anandamide and 2-AG are produced 'on-demand' from cell membrane precursors in a variety of cell types, including both neurons and immune-competent cells in the periphery (B cells, T cells, monocytes/macrophages) and central nervous system (microglia, astrocytes).[20] The induction of 2-AG may be a general response of stellate cells to injury, such as alcohol and hepatitis virus. The endocannabinoid could not be detected in single-culture from replicon cells or LX-2 cells. In this study, we proved that HCV replication may increase 2-AG levels in LX-2 cells, which is most probably due to the increased expression of the gene encoding the 2-AG biosynthetic enzyme DAGLβ and the decreased expression of the genes encoding the 2-AG degradation enzymes MGL and DAGLα. However, previous research has suggested that the increased 2-AG content of stellate cells from ethanol-fed mice is related to the increased biosynthesis of 2-AG but not decreased degradation of 2-AG.[21] This difference requires further exploration.

The liver plays a central role in insulin resistance, reflected in impaired insulin suppression of hepatic glucose production by down-regulating gluconeogenesis and inhibiting glycogenolysis. CREBH, an endoplasmic reticulum stress-associated liver-specific transcription factor, is emerging to be a key player in regulating various hepatic metabolic pathways. It directly regulates the expression of the key hepatic gluconeogenic enzyme genes PEPCK and G6Pase.[22] Previous studies have demonstrated that CREBH is dramatically induced and activated in diet-induced obese rodent models.[23] In this study, we proved that HCV replication activated CB1R and increased CREBH, then up-regulated PEPCK and G6Pase genes and down-regulated glycolytic rate-limiting enzyme GK gene, thus promoting glucose production. AMPK serves as an intracellular sensor for energy homeostasis. The AMPK pathway regulates lipid and glucose metabolism. The increase in AMPK activity reduces glucose production in the liver, whereas it enhances glucose uptake in the skeletal muscle. In hepatic and adipose tissues, CB1 agonist treatment has been demonstrated to inhibit AMPK activity.[24] We proved that HCV replication activated CB1R and decreased AMPK phosphorylation, inhibited cell surface expression of GLUT2, and suppressed cellular glucose uptake. HCV infection might induce high concentrations of glucose in hepatocytes by CB1R–CREBH and CB1R–AMPK signaling pathways, which may confer an advantage in efficient replication of HCV.

The changes mentioned above, including the increase in 2-AG, CB1R expression, and related glucose metabolism genes and glucose metabolism changes, were canceled when the replication of HCV was eliminated by IFN. This strongly suggests that the aforementioned metabolic disorders were selectively suppressed by HCV replication. To clarify the role of CB1R in the improvement of glucose metabolism disorders, we treated replicon cells with special CB1R agonist or antagonist. We proved that the CB1R antagonist could improve glucose metabolism disturbances by an increase in glucose uptake and a decrease in glucose production, and inhibited HCV replication. These findings suggest that endocannabinoids and hepatic CB1 receptors are part of a common pathway involved in the development of glucose metabolism disorders of hepatocytes by HCV infection.

In this study we found the activation of the endocannabinoid system to be associated with glucose metabolism disorders. However data on the endocannabinoid system in CHC patients are lacking, hence we aimed to assess whether circulating and hepatic endocannabinoids are up-regulated. We found that with the increase in HOMA-IR, anandamide concentrations in the peripheral circulation and the level of CB1R in liver samples obtained from CHC patients gradually increased (unpublished data). Based on experimental and therapeutic evidence, hepato-selective CB1R antagonists with far fewer psychiatric side-effects than first-generation CB1R antagonists may be promising for CHC patients, especially for those who present insulin resistance and steatosis. The concomitant administration of these CB1R antagonists with PEG-IFN plus ribavirin therapy is worth studying.

In conclusion, this study proved that HCV replication may not only increase 2-AG content, but may also up-regulate the expression of CB1R of hepatocytes, then change the expression profile of glucose metabolism-related genes, thereby causing glucose metabolism disorders of hepatocytes and promoting HCV replication (Figure 6). Treatment with CB1R blockade improved glucose metabolic disorders and inhibited viral genome replication, suggesting that selective targeting of peripheral CB1R may be effective in anti-HCV therapy for CHC patients with insulin resistance.

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